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Modulation of interleukin-6 by β_2-adrenoceptor in endotoxin-stimulated renal macrophage cells

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Modulation of interleukin-6 by β_2-adrenoceptor in endotoxin-stimulated renal macrophage cells.

Background. Activation of the cAMP signaling pathway by means of β_2-adrenoceptor agonists has been shown to up-regulate interleukin-6 (IL-6) gene expression and to stimulate IL-6 production in macrophage cells. However, whether β_2-adrenoceptor activation can also modify the rate of IL-6 production in macrophage cells activated by the bacterial endotoxins has not yet been determined. Using renal resident macrophage cells treated with endotoxin, lipopolysaccharide (LPS), and β_2-adrenoceptor agonist, terbutaline, we investigated the role of cAMP pathway, tumor necrosis factor (TNF)-α and mitogen-activated protein kinase (MAPK) pathway (p42/p44) in regulating IL-6 production.

Methods. IL-6 protein, mRNA, and promoter activity were measured in these cells exposed to LPS (1 μg/ml) and/or terbutaline (10^{-9} to 10^{-6} M). Furthermore, the time course effects of terbutaline on cAMP, MAPK (p42/p44), and TNF-α release were evaluated in the cells.

Results. Terbutaline at high concentrations (10^{-6} M) significantly up-regulated IL-6 by approximately 25% ($P < 0.05$), whereas at a lower concentration (10^{-8} M), it down-regulated IL-6 production by 42% ($P < 0.05$). Terbutaline (10^{-8} and 10^{-6} M) caused a concentration- and time-dependent stimulation of cAMP ($P < 0.05$) and TNF production ($P < 0.05$) and a time-dependent decrease in MAPK activity ($P < 0.05$). Following the addition of a cAMP inhibitor, IL-6 promoter activity was correlated with TNF-α levels and MAPK activity.

Conclusions. A biphasic effect of β_2-adrenoceptor agonist on IL-6 production in renal resident macrophage cells became apparent when LPS was exposed to the cells. The terbutaline-induced down-regulation of IL-6 gene production was mediated by an inhibitory effect of terbutaline on TNF-α, which was exerted through the MAPK and cAMP pathways, whereas the up-regulation appeared to be due to a direct action of intracellular cAMP.

Key words: bacterial endotoxins, cAMP, glomerulonephritis, renal injury, lipopolysaccharide, ischemia.

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In many types of renal glomerulonephritis, macrophages infiltrate into the glomerulus and interstitium, and this has been taken as being the initial step in inducing renal damage [1]. Furthermore, it has been reported that macrophages are involved in the development of interstitial nephritis and obstructive uropathy [2]. Although the mechanisms mediating the macrophage-induced renal damage remain unclear, the pathophysiological responses in these renal diseases are associated with the production of proinflammatory cytokines [1], which play a role in ischemia and toxic chemical injury within the renal tissue [3].

Among these proinflammatory cytokines, interleukin-6 (IL-6) is a pleiotropic cytokine that is involved in inflammatory and immune responses, acute phase reactions, and hematopoiesis. At the level of the kidney, IL-6 is a key factor in mediating various components of the immune and inflammatory response [4]. For example, IL-6 can induce the adhesion of circulating cells to the glomerular capillary wall [5]. It can induce proliferation of mesangial cells [6] and is associated with mesangial proliferative glomerulonephritis [7]. Therefore, there is a strong possibility that renal IL-6 generation is one of the most important initial steps in the pathophysiology of renal disease and injury [8].

There is increasing evidence that activation of β_2-adrenoceptors can modulate the production of lipopolysaccharide (LPS)-induced inflammatory cytokines, such as tumor necrosis factor (TNF) and IL-1, in some tissues and organs [9, 10], and Hetier et al have suggested that β-adrenoceptor agonists may have an anti-inflammatory influence on the cytokine network during the course of the immunological responses [11]. However, the action of β_2-adrenoceptor stimulation on IL-6 production is quite controversial. Liao et al observed that β_2-adrenoceptor mediated processes increased LPS-induced IL-6 production in liver cells [9], whereas Maimone et al reported that exposure of astrocytes to norepinephrine elevated IL-6, which was mediated predominantly by β_2-

adrenoceptors and the activation of adenylate cyclase [12]. It is recognized that intracellular cAMP plays an important role in the stimulation of IL-6 gene expression [13], and it has been suggested that raised IL-6 production caused by β_2-adrenoceptor activation is mediated through the cAMP pathway. On the other hand, Straub et al demonstrated that isoproterenol inhibited IL-6 secretion in the spleen [14], whereas our own studies also indicated an inhibitory effect of β_2-adrenoceptor activation following LPS-induced IL-6 gene transcription in rat astrocytes [15]. Furthermore, in an *in vivo* study, epinephrine infusion into human subjects did not affect IL-6 production following an LPS challenge [16]. These findings suggest that factors and/or regulatory mechanisms other than the cAMP pathway contribute to β_2-adrenoceptor–mediated IL-6 production. Consequently, the mechanisms by which β_2-adrenoceptor activation determine IL-6 production both normally and in pathophysiological states such as endotoxemia are unclear.

This study was designed to examine the regulatory mechanisms involved in the LPS-induced renal IL-6 production following β_2-adrenoceptor stimulation with terbutaline. Resident renal macrophages were challenged with LPS, and the role of β_2-adrenoceptor–mediated intracellular cAMP in the regulation of IL-6 transcriptional activity was determined. Furthermore, because the rate of IL-6 transcription is also dependent on the generation of TNF-α, we examined the effect of β_2-adrenoceptor stimulation on mitogen-activated protein kinase (MAPK) activity (p42/p44), which, in turn, contributes to the regulation of TNF-α production.

METHODS

Reagents

Dulbecco's modified Eagle's medium (DMEM), glutamine, kanamycin, HEPES, antibody against β_2-adrenoceptor, PD098059, and fibronectin were obtained from Cosmo Bio Corp. (Tokyo, Japan). Genomic DNA isolation kits were obtained from GIBCO BRL (Life Technologies Inc., Gaithersburg, MD, USA). pGV-B2 (Pica Gene basic vector-2) and cell lysis buffer were from Toyo Inc. Corp. (Tokyo, Japan). Nhe I and Hind III were obtained from Takara Corp. (Shiga, Japan). Fetal bovine serum (FBS) was purchased from Dainippon Pharmaceutical Corp. (Tokyo, Japan). Transfectam was supplied by Bio Sepra Inc. (Marlborough, MA, USA). The luminometer (Lumat LB953AT) was purchased from Berthold (Wildbad, Germany). Isogen was obtained from Nippon Gene (Tokyo, Japan), and ICI 118,551 was obtained from Funakoshi (Tokyo, Japan). ABI 377 was supplied by PE-ABI Corp. (Foster City, CA, USA). Rat TNF-α and IL-6 enzyme-linked immunosorbent assay (ELISA) kits were from Biosource International Inc. (Camarillo, CA, USA). The cAMP ELISA kit, MAPK (p42/p44) assay kit, and isotopes ([α-^{32}P]dCTP, [γ-^{32}P]ATP) were purchased from Amersham Japan (Tokyo, Japan). Unless stated, reagents were from Sigma Chemical Co. (St. Louis, MO, USA).

Construction of reporter plasmid

The IL-6 promoter was amplified using Wistar rat genomic DNA as a template, which was extracted from spleen using a genomic DNA isolation kit. The amplification profile of the target DNA was performed as follows: denaturation at 94°C for one minute, primer annealing at 55°C for one minute, and extension at 72°C for two minutes for the first 20 cycles, followed by 30 cycles. For the first polymerase chain reaction (PCR), the upstream primer extended from 1806 to 1825 bp of the rat IL-6 gene [17], whereas the downstream primer extended from 3127 to 3146 bp of the rat IL-6. For the second PCR, the upstream primer extended from 2353 to 2378 bp of the rat IL-6 gene [17] with an engineered restriction for Nhe I at bp 2357, and the downstream primer extended from 2933 to 2954 bp of the rat IL-6 with an engineered restriction for Hind III at bp 2950. The amplified product was electrophoresed on a 1% agarose gel, and a discrete band of the expected size (0.6kb) was isolated from the gel, digested with Nhe I and Hind III, and subsequently cloned into Nhe I-Hind III of the pGV-B2 (Pica Gene basic vector-2) to generate pGV-B2-IL-6prom. This region contains potential recognition sites for several transcription factors, including the nuclear factor (NF)-κB and NF-IL-6, and comparison of the region with human IL-6 promoter revealed a high degree of conservation. Confirmation of the identity of the insert with the known sequence of the promoter was obtained by means of restriction mapping and sequencing. The sequences of these promoters were checked by the cycle sequencing method using an automatic sequencer (ABI 377).

Renal macrophage cells and culture conditions

Primary cultures of resident renal macrophage cells, including glomerular and tubular macrophages, were performed using a modification of the method of Cole and de Vellis [18]. The primary cultures were initiated from whole kidneys pooled from batches of newborn Wistar rats (day 1 and 2), which were euthanized under ether anesthesia. The kidneys were dissected and then minced with a razor blade to yield 1 mm^3 fragments, which were then resuspended gently in DMEM supplemented with 10% FBS, 10 mM HEPES, L-glutamine (600 mg/liter), and kanamycin (100 μg/ml) and were plated into tissue culture flask (75 cm^2) precoated with fibronectin (0.1 mg/ml). The cells were incubated in the flask at 37°C in 5% CO_2. The medium was changed every three days, and after two weeks cells growing on top of the confluent fibroblast monolayer were detached by

overnight shaking. Free cells were collected, and trypan blue was used to assess viability. Cells were replated in 25 cm^2 tissue culture dishes. Macrophage purity was more than 90% as determined by Ia induction by interferon (IFN)-γ using antimouse and rat Ia monoclonal antibody PE conjugate. Furthermore, the cells were identified by means of immunostaining using double labeling with β_2-adrenoceptor antibody-FITC conjugate. For the study for protein, mRNA accumulation, cAMP, and MAPK assay, the cells (1×10^6) were incubated for up to 24 hours with serum-free DMEM (5 ml). For the luciferase assay, three hours before transfection, the cells at 3×10^5 cells were washed with phosphate-buffered saline, and the medium was replaced with fresh medium containing no serum.

Analysis of tumor necrosis factor-α and interleukin-6 protein and mRNA

To estimate the effect of β_2-adrenoceptor stimulation on TNF-α and IL-6 mRNA and protein expression, supernatants were removed for TNF-α and IL-6 protein assay, and the cells were then collected for IL-6 mRNA measurement. Rat TNF-α and IL-6 proteins were estimated using ELISA kits according to the manufacturer's instructions. We estimated mRNA levels using Northern blot hybridization analysis, as described in our previous study [19]. Briefly, total RNA was extracted from the cells using Isogen according to the manufacturer's instructions. For Northern blot hybridization, the 600 bp Eco RI/Hind III fragment derived from the cDNA insert of clone pGEM (a gift from Dr. S. Akira, University of Osaka) for murine IL-6 and the 420 bp Hinf I fragment for human β-actin (National Children's Research Center, Tokyo, Japan) were labeled by means of the oligolabeling method in the presence of [α-^{32}P] dCTP and used as a hybridization probe. All mRNA samples (10 μg) were simultaneously applied to a Biodyne A membrane, hybridized and exposed for the same time. The β-actin cDNA probe was used as a loading control after the IL-6 probe was stripped from the membrane.

Measurement of mitogen-activated protein kinase (p42/p44) and cAMP

Mitogen-activated protein kinase was estimated using a commercial radioimmunoassay kit based on the p42/p44 MAPK-catalyzed transfer of the γ-phosphate group of adenosine 5′-triphosphate to a peptide that is selective for p42/p44 MAPK. Cells were lyzed in 10 mM Tris, 150 mM NaCl, 2 mM EGTA, 2 mM dithiothreitol, 1 mM orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin (pH 7.4) and then centrifuged at 25,000 × *g* for 20 minutes. The supernatant was retained, as it contained the cytoplasmic MAPK. The reaction was initiated by the addition of [γ-^{32}P] ATP at 30°C for 30 minutes, and the rate of incorporation of ^{32}P into the peptide reflected MAPK activity. Intracellular cAMP was estimated using a commercially available ELISA kit in which the assay was based on the competition between unlabeled cAMP and a fixed quantity of peroxidase-labeled cAMP for a limited number of binding sites on a cAMP-specific antibody. Cells were lyzed using a liquid-phase extraction method, and the supernatants were used for the analysis.

Transfection and luciferase assay and experimental protocol

Transfection of pGV-B2-IL-6prom into the renal macrophage cells was performed using a Transfectam kit following the manufacturer's recommendations. The Transfectam stock solution (15 μl) was added to tubes containing 500 μl of complete serum-free DMEM and vortexed. The pGV-B2-IL-6prom (4 μg) and pact β-gal plasmid (1 μg) was constructed and hence contained β-galactosidase activity (gift from Professor M. Yamamoto, The Institute of Medical Science, University of Tokyo, Tokyo, Japan) suspended in 500 μl of complete serum-free DMEM and vortexed. The DNA and Transfectam stock solutions were immediately mixed and then exposed to the cells. The Transfectam/DNA complex was left in contact with the cells for six hours, and then 1 ml of DMEM containing 20% FCS was added to the dishes for three hours. Cells were maintained in serum-free DMEM for 24 hours prior to the experiments.

For the luciferase assay, the cells were incubated for one or three hours with serum-free DMEM under the following conditions. To investigate the effect of the β_2-adrenoceptor agonist terbutaline on the transcriptional activity of the IL-6 gene, terbutaline (10^{-8} or 10^{-6} M) was added with or without the LPS (1 μg/ml) challenge. To establish whether the effects of terbutaline were mediated via β_2-adrenoceptors, the β_2-adrenoceptor antagonist ICI 118,551 was used. To abolish any action of intracellular cAMP, the cAMP inhibitor (H-89, 5×10^{-6} M) was prepared. To study the effect of cAMP on the transcriptional activity of the IL-6 gene, an analogue of cAMP, dpcAMP (10^{-7}, 10^{-5}, or 10^{-3} M), was added to the dishes.

The incubated cells were harvested by adding 400 μl of lysis buffer [10 mM Tris, 10 mM ethylenediaminetetraacetic acid (EDTA), and 150 mM NaCl, pH 8.0] at one or three hours after administration. The luciferase activity was measured using a luminometer (Lumat LB953AT) and a Pica Gene Luciferase assay system. The luciferase activities were normalized on the basis of β-galactosidase activities, which were assayed as described previously [15]. The data were expressed as the fold activation, which was calculated by dividing normalized luciferase activity (luciferase/β-galactosidase) in the cells by the level of activity in the "control." The experiments in which transfection was performed simultaneously were run three times.

Protocol of the study for protein, mRNA accumulation, cAMP, and mitogen-activated protein kinase assay

To investigate the time course effect of the β_2-adrenoceptor agonist on IL-6 and TNF-α levels in the supernatant of rat renal resident macrophage cell cultures, we measured supernatant IL-6 and TNF protein levels at 0, 1, 2, 3, 4, 6, and 24 hours after LPS (1 μg/ml) challenge and/or terbutaline (Ter 10^{-6} to 10^{-9} M). To determine whether the effects of terbutaline were mediated via β_2-adrenoceptors, the β_2-adrenoceptor antagonist (ICI 118,551:ICI) was used. To abolish any action of intracellular cAMP, the cAMP inhibitor (H-89, 5×10^{-6} M) was prepared. We estimated IL-6 mRNA levels at 24 hours after LPS (1 μg/ml) challenge with or without the addition of terbutaline (Ter 10^{-6} or 10^{-8} M) and measured intracellular cAMP levels at one and three hours after the administration of these compounds. MAPK activities were assayed 0, 20, 40, 60, and 80 minutes after LPS (1 μg/ml) challenge either in the absence or presence of terbutaline (Ter 10^{-6} or 10^{-8} M). To examine the influence of MAPK (p42/p44) inhibition on IL-6 and TNF-α releases from the cells, LPS (1 μg/ml) was added to the cells after 20 minutes of pretreatment with MAPK/extracellular signal-regulated kinase-1 (MEK-1) inhibitor, PD098059 (10^{-5} or 10^{-6} M). IL-6 or TNF-α was measured in the supernatant 24 or 3 hours after the LPS challenge, respectively.

Statistics

Statistical analysis was undertaken using analysis of variance followed by the Bonferroni and Dunnet tests or Kruskal Wallis test for multiple comparisons of the luciferase activity. Results were expressed as mean ± SE of the mean.

RESULTS

Effects of β_2-adrenoceptor stimulation on interleukin-6 production

Figure 1 shows the time course of LPS on IL-6 protein supernatant levels of macrophage cell cultures alone and in the presence of the β_2-adrenoceptor agonist terbutaline. Following three hours of exposure to LPS, there was a slight suppression of IL-6 levels, but at 24 hours there was a sharp increase in production of some 45-fold. After a three-hour exposure to the high concentration of terbutaline (10^{-6} M), the increase in IL-6 levels induced by LPS was enhanced, reaching some 57-fold increase at 24 hours, which was significantly greater than that achieved with LPS alone ($P < 0.05$). By contrast, in the cells exposed to the low concentration of terbutaline (10^{-8} M), the LPS-induced increases in IL-6 production were attenuated, achieving only a 33-fold increase at 24 hours ($P < 0.05$). Together, these data indicate that

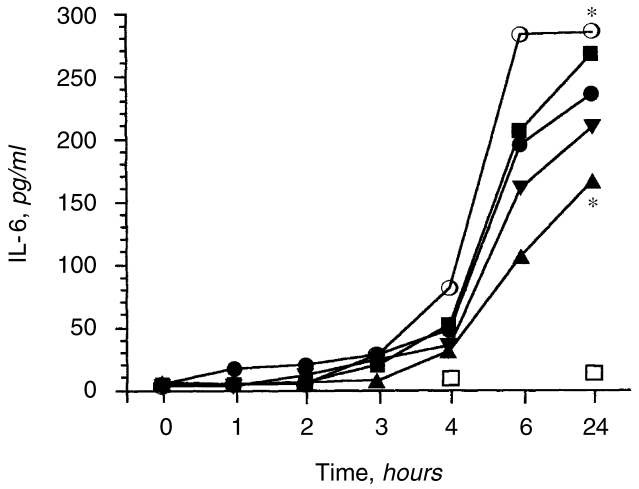

Fig. 1. Time-course of interleukin-6 (IL-6) release from the renal resident macrophage cells following the administration of the β_2-adrenoceptor agonist terbutaline. Cells were incubated in the presence of lipopolysaccharide (LPS; 1 μg/ml, ●) alone, and LPS + terbutaline (Ter 10^{-9} M, ▼; Ter 10^{-8} M, ▲; Ter 10^{-7} M, ■; Ter 10^{-6} M; ○), or without any treatment (control, □) for up to 24 hours. These drugs were added to the cells at the same time. Means for triplicate cultures are shown. *$P < 0.05$ vs. IL-6 level at 24 hours in cells exposed to LPS alone.

terbutaline exerts a biphasic effect on IL-6 production that was dependent on the incubation concentration when the cells were exposed to LPS. When terbutaline (10^{-6} to 10^{-9} M) alone was given, the IL-6 level at 24 hours (terbutaline 10^{-6} M, 21 ± 5 pg/ml; 10^{-7} M, 18 ± 3 pg/ml; 10^{-8} M, 13 ± 3 pg/ml; 10^{-9} M, 14 ± 5 pg/ml) was not changed compared with control (12 ± 4 pg/ml). These findings were supported by observations on the level of cellular IL-6 mRNA (Fig. 2), in that the LPS-induced increase in IL-6 mRNA was significantly ($P < 0.05$) greater in the presence of 10^{-6} M terbutaline but was significantly depressed ($P < 0.05$) following the addition of 10^{-8} M terbutaline. Under control conditions, in which the cells were exposed to terbutaline but without the LPS challenge, IL-6 mRNA expression was not changed. Figure 3 shows the responses in IL-6 promoter activity induced by LPS alone or in combination with terbutaline following one and three hour exposures of the cells. Exposure of the cells for one hour to the LPS increased IL-6 promoter activity by approximately 2.7-fold, but this was significantly ($P < 0.05$) suppressed by terbutaline in a concentration-dependent manner. The addition of terbutaline alone at 10^{-6} and 10^{-8} M did not change IL-6 promoter activity. Interestingly, three hours after the incubation with LPS, IL-6 promoter activity was increased by approximately 14-fold, but when combined with the high concentration of terbutaline (10^{-6} M), the IL-6 promoter activity was significantly ($P < 0.05$) enhanced compared with LPS alone, reaching a 21-fold rise. Coincubation of LPS plus the low concentration of terbutaline (10^{-8} M) significantly ($P < 0.05$) depressed

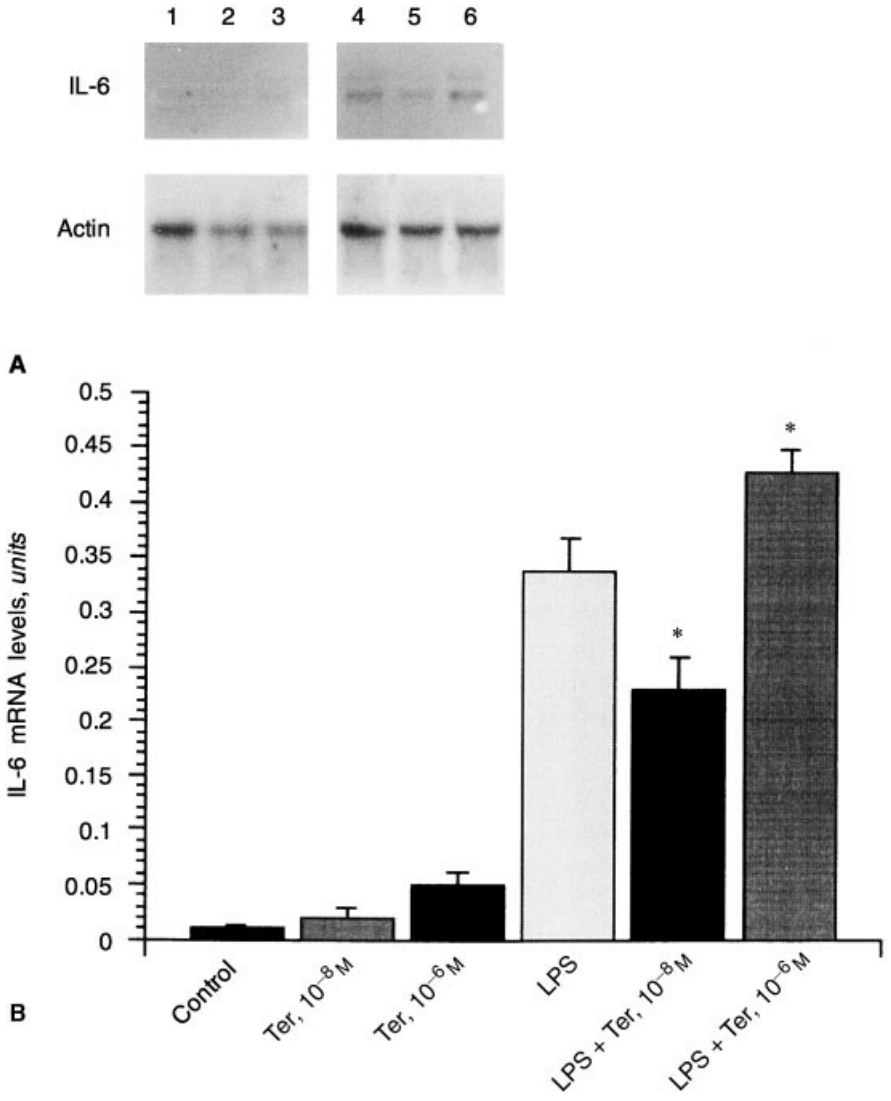

Fig. 2. Northern blot analysis of representative interleukin-6 (IL-6) and β-actin (actin) mRNA and graph of changes in lipopolysaccharide (LPS)-induced IL-6 mRNA units (IL-6/β-actin mRNA) from the renal resident macrophage cells in the presence and absence of the β_2-adrenoceptor agonist terbutaline. Cells were incubated in the presence of LPS (1 μg/ml) or LPS plus terbutaline (Ter 10^{-6} and Ter 10^{-8} M) for 24 hours. Numbers in (A) are: (1) control; (2) Ter 10^{-8} M; (3) Ter 10^{-6} M; (4) LPS; (5) LPS + Ter 10^{-8} M; (6) LPS + Ter 10^{-6} M. Means ± SE for triplicate cultures are shown. *$P < 0.05$ vs. cells exposed to LPS alone.

the LPS-induced promoter activity, achieving only a ninefold increase. These changes in IL-6 promoter activity closely corresponded to the pattern of responses observed in the protein and mRNA levels of IL-6. Under the conditions in which the LPS challenge was not given, terbutaline increased IL-6 promoter activity in a dose-dependent manner, although by only approximately 70% (10^{-6} M). The increases in IL-6 promoter activity induced by the LPS plus the terbutaline were abolished using the β_2-adrenoceptor antagonist ICI 118,551, compatible with β_2-adrenoceptors mediating these changes in IL-6 promoter activity in the cells; that is, not only was there potentiation by the high concentration of terbutaline but also attenuation by the low concentration of terbutaline.

Intracellular cAMP and interleukin-6 promoter activity

Figure 4 shows the effect of the cAMP analogue on IL-6 promoter activity in the cells one hour after administration. It is clear that dpcAMP stimulated IL-6 promoter activity in a concentration-related fashion, reaching an approximate sixfold ($P < 0.05$) stimulation at 10^{-3} M dpcAMP. Figure 5 examines the changes in endogenous concentration of intracellular cAMP in the cells exposed to LPS alone or together with terbutaline. Terbutaline 10^{-6} and 10^{-8} M significantly ($P < 0.05$) increased intracellular cAMP levels in a concentration-dependent manner, and the pattern of these responses was unaffected by the presence of LPS. To further evaluate the contribution of intracellular cAMP to the IL-6 promoter activity induced by terbutaline administration, an inhibitor of the cAMP-protein kinase A (PKA) pathway, H-89, was added to the cells treated with LPS and/or terbutaline; the responses are shown in Figure 6. Following a one-hour exposure to H-89, the LPS increased IL-6 promoter activity by some 3.5- to 4-fold, but this response was significantly ($P < 0.05$) attenuated by terbutaline in a

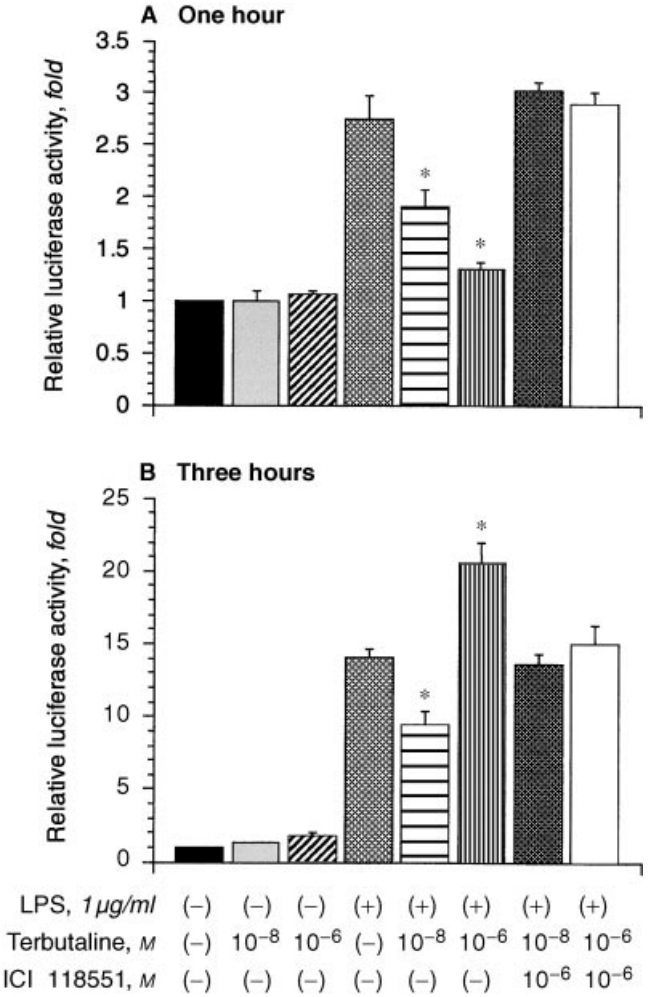

Fig. 3. Luciferase activities expressed by pGV-B2-IL-6prom (IL-6 promoter construct) in renal resident macrophage cells. Cells were cultured in the presence or absence of terbutaline (10^{-6} M, 10^{-8} M), ICI 118,551 (10^{-6} M), or LPS (1 μg/ml), after which cytosolic extracts were prepared and used for the luciferase and β-gal assays. The fold activation is the ratio of normalized luciferase activity (luciferase/β-galactosidase) divided by the level of the normalized activity in the cells untreated with any regimens. Data are the mean ± SE from three experiments. *$P < 0.05$ vs. cells exposed to LPS alone.

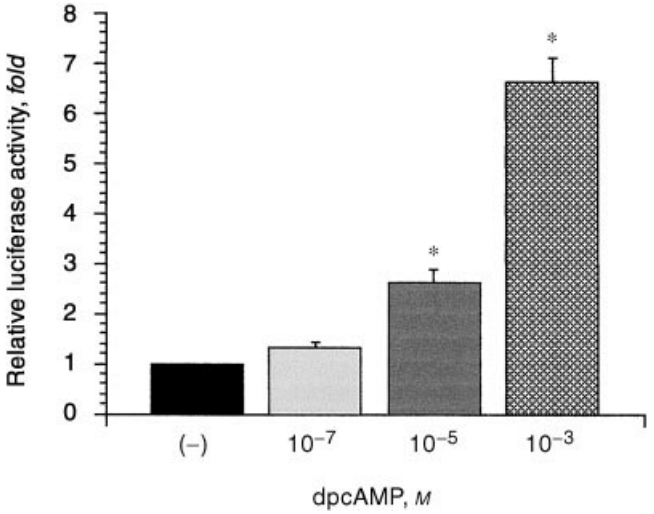

Fig. 4. Luciferase activities expressed by pGV-B2-IL-6prom (IL-6 promoter construct) in the renal resident macrophage cells. Cells were cultured for one hour in the presence or absence of dpcAMP (10^{-7} to 10^{-3} M), after which cytosolic extracts were prepared and used for the luciferase and β-gal assays. The fold activation is the ratio of normalized luciferase activity (luciferase/β-galactosidase) divided by the level of the normalized activity in the untreated cells. Data are the mean ± SE from three experiments. *$P < 0.05$ vs. cells exposed to dpcAMP (10^{-7} M).

concentration-dependent manner by approximately 30 and 55% at 10^{-8} and 10^{-6} M (both $P < 0.05$), respectively, which was similar to the pattern of responses obtained in the absence of H-89 (Fig. 3). However, at three hours in the presence of H-89, the LPS-induced stimulation of IL-6 promoter activity reached some 15-fold but was unaffected by the 10^{-8} M terbutaline and was depressed by 24% ($P < 0.05$) in the presence of 10^{-6} M terbutaline. Importantly, this pattern of response was very different from the biphasic concentration-response observed in the absence of H-89, as shown in Figure 3. Exposure of the cells to terbutaline alone had very little influence on IL-6 promoter activity after either one or three hours, being comparable to that found when H-89 was not present (Fig. 3). These findings indicate that the LPS-induced stimulation of IL-6 promoter activity and its modulation by β_2-adrenoceptor activation seem to involve not only intracellular cAMP but some other factors.

Suppression of MAPK (p42/p44) by terbutaline

Figure 7A shows the time course of action of terbutaline on MAPK (p42/p44) activity in the cells. MAPK activity was stimulated by the LPS challenge, beginning some 20 minutes after the initial exposure, and reached a peak level at 40 minutes, of some 2.3-fold, and then returned to the baseline at 80 minutes. In the presence of terbutaline, the peak level of LPS-induced MAPK activity at 40 minutes was suppressed. In Figure 7B, the comparisons of MAPK activity at 40 minutes between these groups are shown as bar graphs. Terbutaline (10^{-6} and 10^{-8} M) significantly ($P < 0.05$) suppressed MAPK activity induced by the LPS. The terbutaline suppression of LPS-induced MAPK activity was abolished by the β_2-adrenoceptor antagonist ICI 118,551, which was most likely caused by functional β_2- adrenoceptor activation.

Suppression of tumor necrosis factor-α by terbutaline

Figure 8 presents the changes in supernatant levels of TNF-α in cells treated with LPS alone or in combination with terbutaline. At one and three hours after exposure to LPS, TNF-α levels were stimulated approximately 3- and 23-fold, respectively, and these responses were significantly ($P < 0.05$) suppressed in a concentration-related way by the terbutaline at both time points. How-

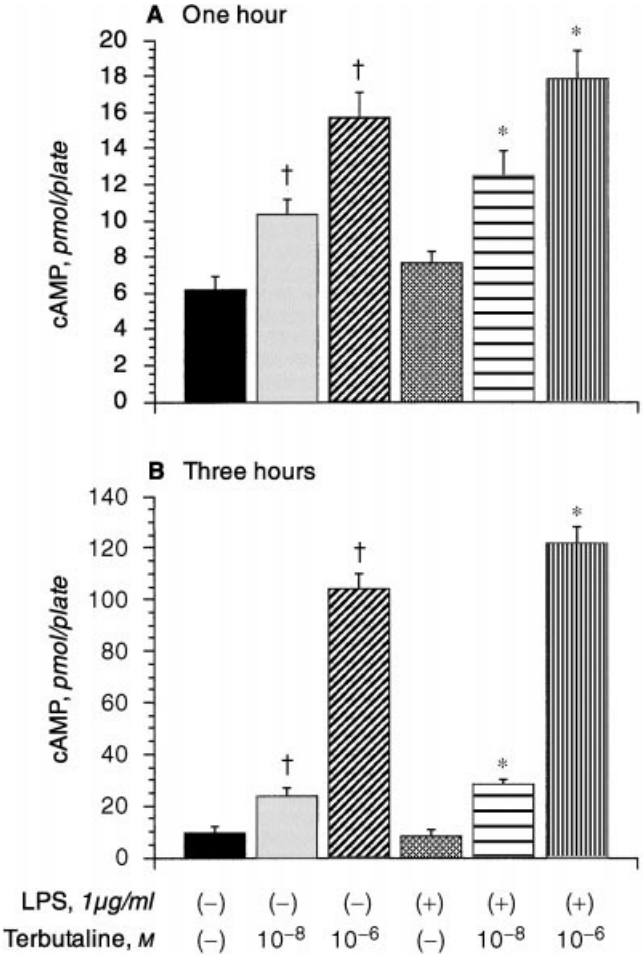

Fig. 5. Effects of terbutaline on intracellular cAMP concentrations in renal resident macrophage cells at one (*A*) and three (*B*) hours after administration are shown as pmol/plate (10^6 cells). Cells were incubated in the presence or absence of terbutaline (10^{-6} M, 10^{-8} M) or lipopolysaccharide (LPS; 1 μg/ml) or without any treatment. Means of triplicate cultures are shown. *$P < 0.05$ vs. cells exposed with LPS alone. †$P < 0.05$ vs. cells without any treatment.

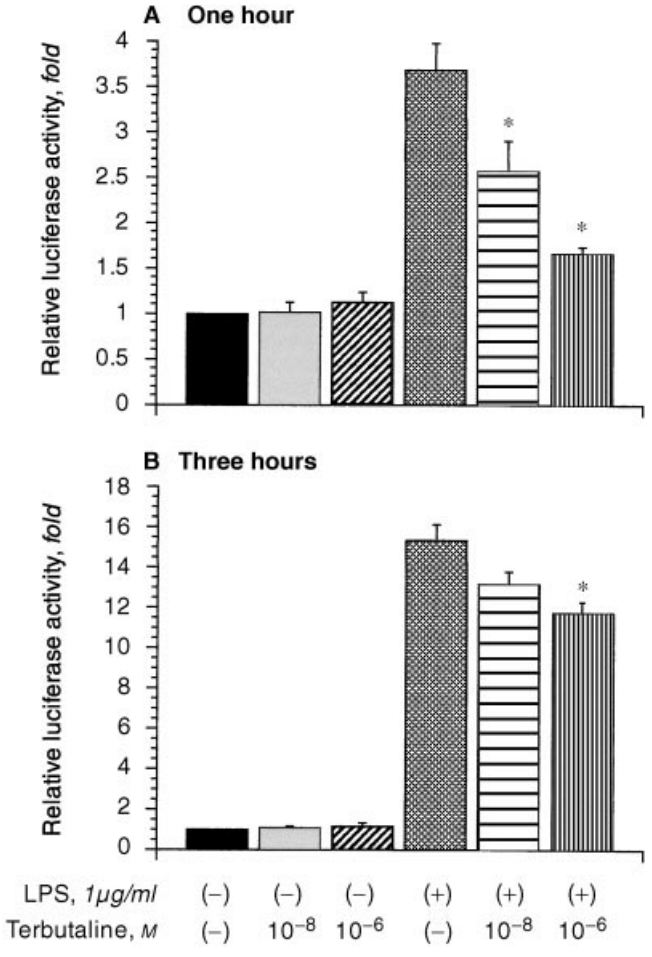

Fig. 6. Luciferase activities expressed by the pGV-B2-IL-6prom (IL-6 promoter construct) in renal resident macrophage cells. After the cells were exposed to the cAMP inhibitor, H-89 (5×10^{-6} M), they were cultured in the presence or absence of terbutaline (10^{-6} M, 10^{-8} M) or lipopolysaccharide (LPS; 1 μg/ml) for one (*A*) and three (*B*) hours, after which cytosolic extracts were prepared and used for the luciferase and β-gal assays. The fold activation is the ratio of normalized luciferase activity (luciferase/β-galactosidase) divided by level of the normalized activity in the untreated cells. Data are the mean ± SE from three experiments. *$P < 0.05$ vs. cells exposed to LPS alone.

ever, in the cells exposed to terbutaline alone, the TNF-α level was not changed at either time point. In Figure 9, in the presence of the cAMP inhibitor, H-89, LPS-induced TNF-α level was significantly suppressed by terbutaline at both time points. The magnitude of the inhibitory response at three hours was small (terbutaline 10^{-6} M, 22% inhibition) compared with that found when H-89 was not present (10^{-6} M, 51% inhibition; Fig. 8). Importantly, suppression of TNF-α by terbutaline partially involved the cAMP pathway, but it was also influenced by other mechanisms.

Suppression of interleukin-6 and tumor necrosis factor-α release by MEK-1 inhibitor

The potential role of the MAPK (p42/p44) in LPS signal transduction on IL-6 and TNF-α releases was assessed using MEK-1 inhibitor, PD098059. The inhibitor significantly ($P < 0.05$) decreased IL-6 and TNF-α release from the cells following LPS stimulation (Table 1). Inhibition of the MAPK (p42/p44) pathway resulted in a block of the increase in IL-6 and TNF-α production in the cell.

DISCUSSION

Interleukin-6 is a multifunctional cytokine whose activities include stimulation of B-cell differentiation, supporting the growth of hybridoma cell lines, and stimulation of acute phase protein production by hepatocytes. It is recognized that IL-6 can be produced by a variety of cells, such as T cells, B cells, monocytes, fibroblasts, and endothelial cells [4], whereas in the kidney, IL-6 is

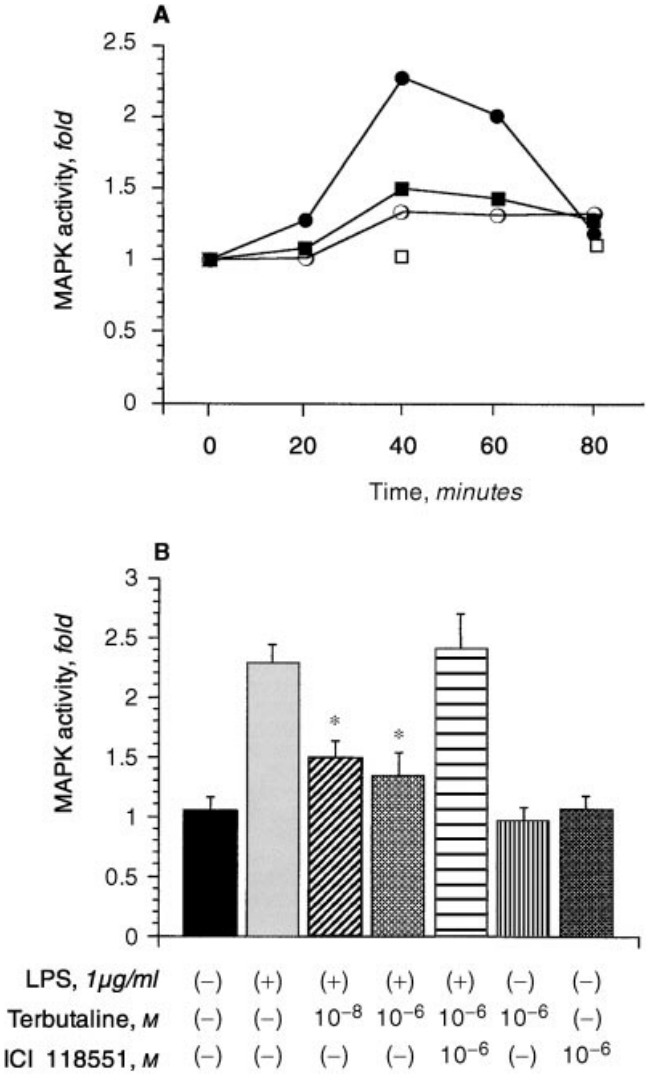

Fig. 7. (*A*) Effect of terbutaline on mitogen-activated protein kinase (MAPK) activities (p42/p44) in renal resident macrophage cells stimulated with LPS and the time-course changes are shown. Cells were incubated in the presence or absence of terbutaline (Ter 10^{-6} M, ○; 10^{-8} M, ■) or LPS (1 μg/ml, ●) or without any treatments (control, □), for up to 90 minutes. The data are expressed as relative changes (fold) to level at time zero in each group. The means of triplicate cultures are shown. (*B*) Effect of terbutaline on MAPK in the cells at 40 minutes after LPS challenge. The data are expressed as relative changes (fold) to level at time zero in each group. ICI 118,551 (10^{-6} M) was used as a β_2-adrenoceptor antagonist. Data are the mean ± SE from three experiments. $^*P < 0.05$ vs. cells exposed to LPS alone.

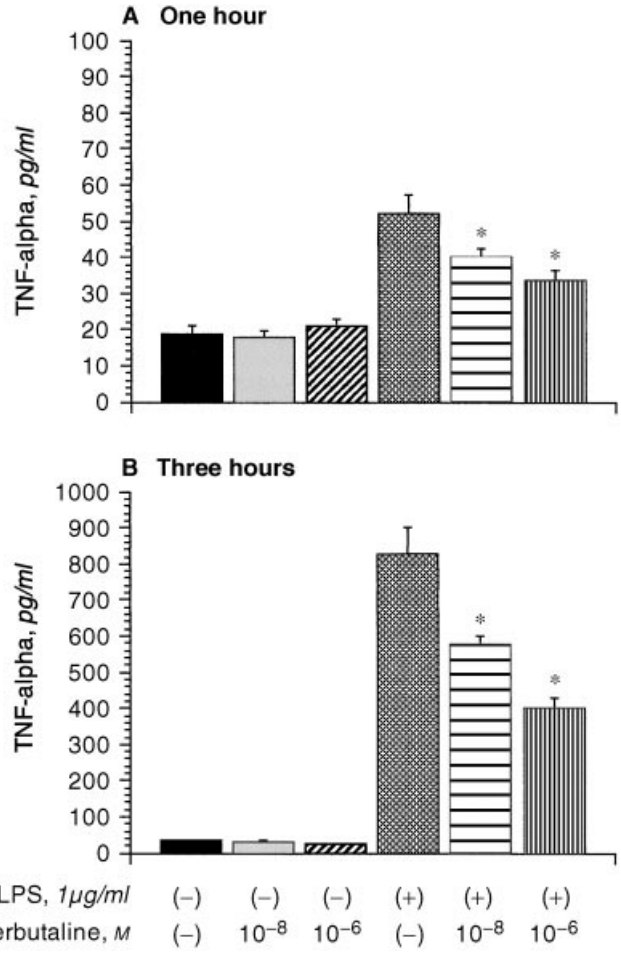

Fig. 8. Effect of β_2-adrenoceptor stimulation on TNF-α release from renal resident macrophage cells. Cells were cultured for one or three hours in the presence or absence of lipopolysaccharide (LPS; 1 μg/ml) or terbutaline (10^{-6} M, 10^{-8} M). Means ± SE of three experiments are shown. $^*P < 0.05$ vs. cells exposed to LPS alone.

produced by mesangial and vascular wall smooth muscle cells [20]. The production of IL-6 can be regulated by TNF-α and infection, whereas enhanced IL-6 gene expression has been observed in autoimmune diseases [4, 21–23]. The renal resident macrophage cells can be activated by LPS and also produce IL-6, as has become evident from the findings of this study, which is compatible with the report that IL-6 increases in states of endotoxemia and may be involved in inducing renal tissue damage [8, 22].

The important finding arising from this study was that LPS was able to cause a time-dependent increase in IL-6 protein production by rat renal resident cells. Moreover, the magnitude of this response was modulated by the β_2-adrenoceptor agonists in a biphasic concentration-dependent way; that is, at high doses, the production of IL-6 was enhanced, whereas at low doses, it was suppressed. It was evident that this biphasic adrenergic modulation of IL-6 was exerted at the mRNA level, as the increases in IL-6 mRNA in response to LPS were greater at the high concentrations of terbutaline and less at low concentrations of terbutaline compared with that generated with LPS alone. The question arose as to whether modulation of the rate of transcription might be responsible for the biphasic response to the β_2-adrenoceptor–mediated effect. It was apparent that a complex relationship existed, in that following a one-hour exposure to terbutaline, there was a concentration-dependent sup-

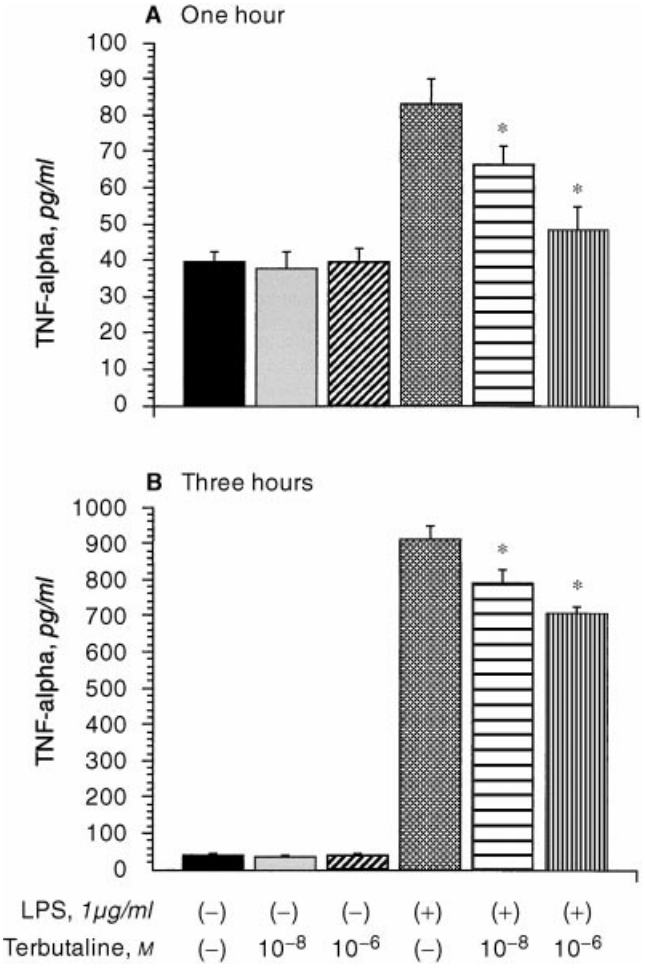

Fig. 9. Effect of β_2-adrenoceptor stimulation on TNF-α release from renal resident macrophage cells following inhibition of the cAMP pathway. After the cAMP inhibitor H-89 (5×10^{-6} M) was added to the dishes, the cells were cultured for one (*A*) or three (*B*) hours in the presence or absence of LPS (1 μg/ml) or terbutaline (10^{-6} M, 10^{-8} M). Means ± SE of three experiments are shown. *$P < 0.05$ vs. cells exposed to LPS alone.

Table 1. Modulation of LPS-induced IL-6 and TNF-α release from rat resident macrophage cells by an MEK-1 inhibitor (PD098059)

	IL-6	TNF-α
	pg/ml	
Control	16 ± 5	31 ± 5
LPS	223 ± 8	775 ± 31
LPS + PD098059 10^{-6} M	182 ± 5[a]	591 ± 32[a]
LPS + PD098059 10^{-5} M	149 ± 10[a]	347 ± 31[a]

Lipopolysaccharide (LPS 1 μg/ml) was added in the cells after 20 minutes of pre-treatment with PD098059. Interleukin (IL)-6 or tumor necrosis factor (TNF)-α released during the following 24 or 3 hours was measured by ELISA, respectively. The results were the means ± SE of three separate experiments.
[a] $P < 0.05$ vs. LPS

pression in the IL-6 promoter activity to LPS, but at three hours, there was an enhanced response at the high and a depressed response at the low concentration of terbutaline. Although both patterns of responses were mediated by β_2-adrenoceptors, as they were blocked by the selective antagonist ICI 118,551, it was clear that different mechanisms were coming into play at different times.

These findings were intriguing in that β_2-adrenoceptor activation causes the generation of the second-messenger cAMP and suggested that it might have multiple effects on the rate of transcription of the IL-6 in response to LPS. This issue was addressed by examining the action of cAMP on IL-6 transcriptional activity, and indeed, there was a very clear and sensitive stimulation of the gene by the second messenger. Moreover, it was evident that the ability of the β_2-adrenoceptor agonist to increase cellular cAMP levels was quite independent of the LPS challenge, again suggesting that the second messenger might be acting in more than one way to influence the IL-6 gene expression. This view was reinforced by the findings of the study using the cAMP inhibitor H-89. The results showed that there was a concentration-related inhibition of the LPS stimulation of IL-6 promoter activity, which was markedly different from the biphasic pattern of response, which was evident when the direct effect of cAMP was investigated. Thus, it is clear that when the cells are exposed to LPS, a range of factors and/or other regulatory mechanisms are brought into play, including the cAMP pathway, which would ultimately determine the rate at which transcription of the IL-6 gene would occur.

One important and potent factor that is strongly and specifically induced by LPS is TNF-α, and its biosynthetic regulation seems to be exerted at several levels. The signaling pathway used by LPS to induce TNF-α biosynthesis is believed to be dependent on the activation of tyrosine kinases [24]. Tyrosine kinase-initiating processes often involve the subsequent activation of the MAPK signaling pathway, and one of the initial LPS-stimulated events is the phosphorylation and activation of the p42/p44 isoforms of MAPK (p42/p44) pathway [25]. In this study, an enhanced MAPK (p42/p44) activity was detected within 20 minutes of cellular stimulation, which was short lived, and the activity decreased to a basal level within 80 minutes, which was compatible with previous reports [26]. Furthermore, we were able to show that suppression of MAPK (p42/p44) pathway using PD098059 resulted in a decreased release of IL-6 and TNF-α from the cells. These observations support an earlier report [27] that suppression of MAPK (p42/p44) pathway diminished LPS-induced IL-6 mRNA and IL-6 production in liver Kupffer cells. The findings of this study extended these observations and demonstrated that β_2-adrenoceptor activation suppressed MAPK (p42/p44) pathway in the cells, which, in turn, was correlated with a decreased TNF-α levels and reduction in IL-6 production. Vanden-Berghe et al indicated that the NF-κB was involved in the regulation of IL-6 gene expression

by TNF-α [28]. Following a one-hour exposure to terbutaline, the LPS-induced IL-6 promoter activity was suppressed and correlated with the decreases in MAPK activity and TNF-α. The effect of terbutaline on IL-6 promoter activity was not influenced by the addition of H-89, which would suggest that the decrease in LPS-induced IL-6 promoter activity observed at one hour after treatment of terbutaline did not rely on intracellular cAMP and might be partially caused by inhibition of TNF-α production as a consequence of the suppression of the MAPK (p42/p44) pathway.

Lipopolysaccharide-induced TNF-α production itself was also depressed by β_2-adrenoceptor activation, but importantly, this was independent of the intracellular cAMP pathway. Previous investigators have reported that the inhibition of TNF-α production by β_2-adrenoceptor activation was dependent on an increase in intracellular cAMP levels [10, 11, 29]. Olivier et al found that an increase in intracellular cAMP concentration decreased the NF-κB–mediated function of TNF-α gene transcription and IL-6 production [30]. This suggests that the cAMP pathway leads to an inhibition of TNF levels, which indirectly would decrease IL-6 production, whereas a direct action of the intracellular cAMP would be to increase IL-6. This direct action was clearly shown by the addition of the cAMP inhibitor H-89, which abolished the stimulatory effect of terbutaline on IL-6 promoter activity after three hours. Therefore, the enhanced IL-6 gene expression in the presence of terbutaline is dependent on the cAMP pathway. The findings suggest that LPS and cAMP-elevating neuropeptides synergistically induce IL-6 production in the cells, as has been observed in previous reports [12, 31]. Conversely, the depressed IL-6 response to LPS following exposure to terbutaline for three hours could not be abolished completely by the cAMP inhibitor H-89. Moreover, TNF-α levels remained low in spite of the addition of H-89, which suggests that factors other than cAMP are involved in the suppression of TNF-α by terbutaline. This view is supported by the report of Seldon et al, who reported that the inhibitory effects of β_2-adrenoceptor agonists on TNF-α gene transcription and/or translation were regulated by a cAMP/cAMP-dependent protein kinase (PKA) cascade and cAMP-independent mechanisms [32].

In summary, this study demonstrates that β_2-adrenoceptor activation with terbutaline stimulated IL-6 transcriptional activity through a PKA-cAMP pathway, and at the same time, β_2-adrenoceptor activation with terbutaline suppressed IL-6 transcriptional activity via an inhibitory effect of cAMP on LPS-induced TNF-α production as well as LPS-induced MAPK (p42/p44) activity. Therefore, the modulation of LPS-induced IL-6 levels by β_2-adrenoceptors depends on the balance between a direct effect of cAMP as a stimulator of IL-6 and an indirect action of TNF-α as a suppresser of IL-6 through cAMP and/or MAPK (p42/p44) pathways. Furthermore, other mechanisms and intracellular signals may be also involved with the modulation of LPS-induced IL-6 production by β_2-adrenoceptor activation.

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APPENDIX

Abbreviations used in this article are: DMEM, Dulbecco's modified Eagle's medium; IL-6, interleukin-6; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MEK-1, MAPK/extracellular signal-regulated kinase-1; NF, nuclear factor; PKA, protein kinase A; TNF, tumor necrosis factor.

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